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14. ABSTRACT

Sandfly fever group viruses in the genus Phlebovirus (family Bunyaviridae) are widely distributed across the globe and are a cause of disease in military troops and indigenous peoples. We assessed the laboratory sensitivity and specificity of the Sand Fly Fever Virus Antigen Assay, a rapid dipstick assay designed to detect sandfly fever Naples virus (SFNV) and Toscana virus (TOSV) against a panel of phleboviruses. The assay detected SFNV and TOSV, as well as other phleboviruses including Aguacate, Anahanga, Arumowot, Chagres, and Punta Toro viruses. It did not detect sandfly fever Sicilian, Heartland, Rio Grande, or Rift Valley fever viruses. It did not produce false positive results in the presence of uninfected sand flies (Lutzomyia longipalpis) or Cache Valley virus, a distantly related bunyavirus. Results from this laboratory evaluation suggest that this assay may be used as a rapid field-deployable assay to detect sand flies infected with TOSV and SFNV, as well as an assortment of other phleboviruses.

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Phlebovirus, Toscana virus, sandfly fever, assay, dipstick

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OPERATIONAL NOTE

LABORATORY VALIDATION OF THE SAND FLY FEVER VIRUS ANTIGEN ASSAY

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ABSTRACT. Sandfly fever group viruses in the genus *Phlebovirus* (family Bunyaviridae) are widely distributed across the globe and are a cause of disease in military troops and indigenous peoples. We assessed the laboratory sensitivity and specificity of the Sand Fly Fever Virus Antigen Assay, a rapid dipstick assay designed to detect sandfly fever Naples virus (SFNV) and Toscana virus (TOSV) against a panel of phleboviruses. The assay detected SFNV and TOSV, as well as other phleboviruses including Aguacate, Anahanga, Arumowot, Chagres, and Punta Toro viruses. It did not detect sandfly fever Sicilian, Heartland, Rio Grande, or Rift Valley fever viruses. It did not produce false positive results in the presence of uninfected sand flies (*Lutzomyia longipalpis*) or Cache Valley virus, a distantly related bunyavirus. Results from this laboratory evaluation suggest that this assay may be used as a rapid field-deployable assay to detect sand flies infected with TOSV and SFNV, as well as an assortment of other phleboviruses.

KEY WORDS *Phlebovirus*, Toscana virus, sandfly fever, assay, dipstick

The sandfly fever (SF) virus group (*Bunyaviridae*: *Phlebovirus*) is composed of arboviruses primarily transmitted by phlebotomine sand flies in the genera *Lutzomyia* and *Phlebotomus* (Diptera: Psychodidae), although some, like Rift Valley fever virus (RVFV) and Arumowot virus, are transmitted by mosquitoes (Tesh 1988). The most clinically significant members of the SF virus group are RVFV, Toscana virus (TOSV), sandfly fever Sicilian virus (SFSV), sandfly fever Naples virus (SFNV), and Punta Toro virus (Tesh 1988, Alkan et al. 2013). These viruses pose a threat to US military personnel and indigenous populations in tropical and temperate regions (Brett-Major and Claborn 2009). In particular, TOSV (a serotype of SFNV) is one of the primary causes of SF disease among US troops in the Mediterranean (Brett-Major and Claborn 2009) and is a common cause of meningitis in Mediterranean and southern European countries during the vector season (Braito et al. 1997). Sandfly fever Sicilian virus also causes sporadic epidemics of Pappataci fevers in humans (Brett-Major and Claborn 1997). Rift Valley fever virus and Arumowot virus are transmitted by mosquitoes, and RVFV can cause serious hemorrhagic disease or high fever in humans (Tomori and Fabiyi 1976, Tesh 1988); RVFV is of particular military concern because it could be used as a biological weapon (Dudley and Woodford 2002). SF viruses are found in the New World, including Punta Toro, Rio Grande, Augacate, Anhanga, and Chagres viruses, and several of these are also known to cause serious disease such as high fever and joint pain (Tesh 1988). Chagres virus has caused human disease in residents of Panama (Srihongse and Johnson 1974) and has also been isolated from US military personnel stationed there (Peralta et al. 1965).

Rapid field assessments of sand flies for phleboviruses have been previously unavailable. The available tests are virus isolation or reverse transcriptase polymerase chain reaction (RT-PCR), which require appropriately equipped and staffed laboratories and several days to weeks to receive test results. The Sand Fly Fever Virus Antigen (SFFVA) Assay (Product number SFVA-K020; VecTOR Test Systems Inc., Thousand Oaks, CA) is a dipstick assay that is easy to perform and interpret and has the potential to be field deployable. Ideally this assay could be used worldwide if it detects multiple strains of SSFV. Samples are processed in a proprietary grinding solution provided with the kit that facilitates wicking of viral antigens up the assay stick. The dipstick is added to an aliquot of the homogenized sample supernatant and allowed to incubate for 15 min. The appearance of a red band at both the test and control zones indicates a positive result, whereas the appearance of a red band at only the control zone indicates a negative result (Fig. 1). All SFFV positives react in the same location on the dipstick indicating a positive but not allowing for virus differentiation. The appearance of no bands at all indicates test failure,

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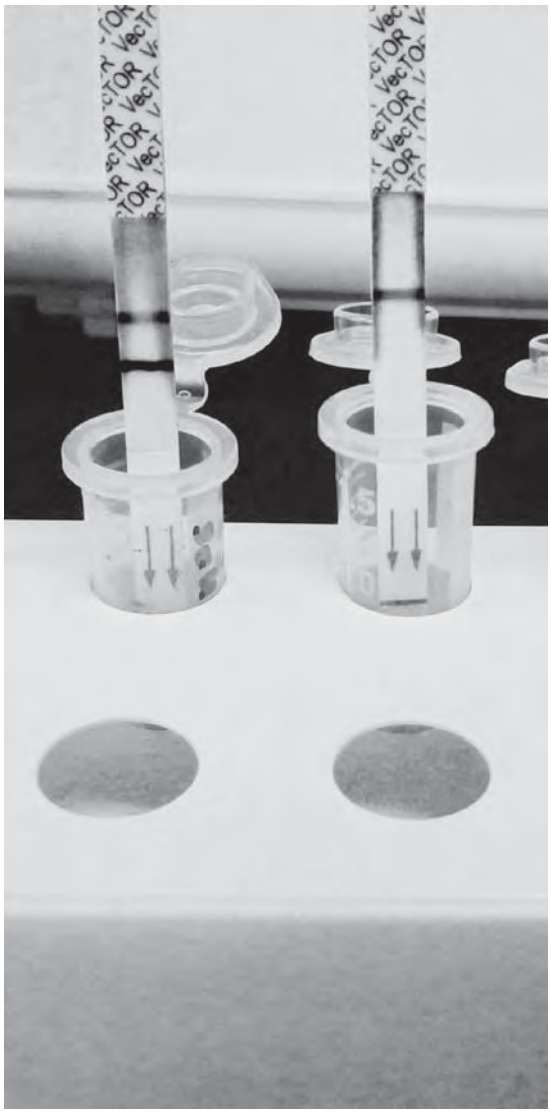

Fig. 1. Positive (left) and negative (right) results on the Sand Fly Fever Virus Antigen Assay dipsticks using Toscana virus and *Lutzomyia longipalpis*.

and a retest of the sample is required. The SFFVA assay was designed, because of a Department of Defense–funded priority, to detect TOSV and SFNV in field-collected sand flies by serologically detecting the partially conserved viral N protein. We evaluated its ability to detect other phleboviruses in the SF group. The SFFVA is analogous to several commercially available assays from VecTOR Test Systems Inc. for malaria, West Nile virus, Rift Valley fever virus, dengue, chikungunya, and several other arboviruses. These assays were described by Ryan et al. (2003).

We conducted a laboratory evaluation of the SFFVA dipstick assay on an assortment of antigenically distinct SF viruses. These included the Old World viruses: TOSV, SFNV, and SFSV. We selected several New World SF viruses including Rio Grande virus, which is the only known phlebovirus transmitted by sand flies in the continental USA (Endris et al. 1983), and Aguacate, Anahanga, Chagres, and Punta Toro viruses from Central and South America, which were chosen based on previously described human infections and published antigenic differences between viral strains (Peralta et al. 1965, Sather 1970, Srihongse and Johnson 1974, Tesh et al. 1975).

We also tested the SFFVA against arthropod-borne phleboviruses that are not members of the SF virus group (Heartland viruses) or are members of the SF virus group but not known to be transmitted by sand flies (RVFV and Arumowot virus). Heartland virus (HRTV) is a North American phlebovirus known to cause febrile illness in people (McMullan et al. 2012) and was recently shown to be transmitted by ticks (Savage et al. 2013). We tested Cache Valley virus (*Bunyaviridae*: *Orthobunyavirus*) as a negative virus control because it is a distantly related genus of *Bunyaviridae*. Finally, uninfected pools of sand flies, *Lutzomyia longipalpis* Franca (Diptera: Psychodidae), were used to confirm that the test strips would not produce false positive results in the presence of homogenized insects.

Two lots of SFFVA were purchased from VecTOR Test Systems. Viruses were purchased from the American Type Culture Collection (Manassas, VA) or were provided by the Centers for Disease Control and Prevention or US Department of Agriculture. Viruses were propagated and titered using VERO cells. Dead *L. longipalpis* were acquired from colonies maintained at the Walter Reed Army Institute of Research (Silver Springs, MD). A voucher specimen was deposited at the Museum of Biological Diversity, Columbus, OH.

Negative controls consisted of either 250 µl of uninfected VectorTest grinding solution, pools of 25 uninfected homogenized *L. longipalpis* in 200 µl of grinding solution, or Cache Valley virus stock diluted in grinding solution to a final volume of 250 µl (Table 1). All other virus samples were produced by serially diluting each virus in VectorTest grinding solution in a final volume of 250 µl. Samples containing TOSV, RVFV, Arumowot, and all New World SF group viruses also contained 25 homogenized *L. longipalpis*. Sandfly fever Sicilian virus, SFNV, and HRTV samples did not contain sand flies, as they were unavailable at the time of testing. Positive and negative results were interpreted according to the presence of 1 or 2 bands as described above (Fig. 1). To control for possible bias, the dipsticks were examined and scored positive or negative by

Table 1. Results of the laboratory evaluation of the Sand Fly Fever Virus Antigen dipstick assay.[1]

Virus	Strain	Titer
Detected by the SFFVA Assay[2]		
Toscana virus	Unknown	$10^{2.4}$ $TCID_{50}$/ml
Sandfly fever Naples virus	Sabin	$10^{3.5}$ PFU/ml
Aguacate virus	VP 175 A	$10^{3.9}$ $TCID_{50}$/ml
Anhanga virus	Be An 46852	$10^{5.5}$ $TCID_{50}$/ml
Arumowot virus	AR 1284-64	$10^{6.1}$ $TCID_{50}$/ml
Chagres virus	JW 10	$10^{6.7}$ $TCID_{50}$/ml
Punta Toro virus	Unknown	$10^{5.9}$ $TCID_{50}$/ml
Not detected by the SFFVA Assay[3]		
Sandfly fever Sicilian virus	Sabin	$10^{5.2}$ PFU/ml
Heartland virus	MO12-75	10^{7} PFU/ml
Rift Valley fever virus	MP12	$10^{7.9}$ $TCID_{50}$/ml
Rio Grande virus	Unknown	$10^{6.5}$ $TCID_{50}$/ml
Cache Valley virus	89B-7060	$10^{6.6}$ $TCID_{50}$/ml

[1] PFU, plaque-forming unit; SFFVA, SandFly Fever Virus Antigen; TCID, tissue culture infectious dose.
[2] Sensitivity results are presented as the lowest detectable titers of viruses that tested positive with the dipsticks.
[3] Specificity of the assay is demonstrated by viruses that were undetected; highest titer tested.

a student who had no knowledge of the contents of each tube and who was uninvolved with any other aspect of the study.

Results of the SFFVA are listed for each virus tested (Table 1). None of the negative controls produced positive results. No positive results were detected for HRTV, RVFV, Rio Grande virus, or SFSV. Failure to detect RVFV was expected, because this virus is not closely related to Toscana virus (Charrel et al. 2009). Rio Grande virus was characterized as a phlebovirus by serology, but its phylogenetic relationship to other *Phlebovirus* is unknown (Calisher et al. 1977). Heartland virus is a tick-borne phlebovirus and thus a distant relative. The SFFVA dipstick assay did not detect SFSV, which is antigenically different from SFNV (Sabin 1955).

All of the other phleboviruses produced positive SFFVA dipstick assay results (Table 1). The assay detected a minimum titer of $10^{2.4}$ tissue culture infectious dose $(TCID)_{50}$/ml of TOSV or a minimum titer of $10^{3.5}$ plaque-forming units (PFU)/ml of SFNV. Aguacate, Anahanga, Arumowot, Chagres, and Punta Toro viruses produced positive bands; their respective sensitivity limits are listed in Table 1. Bands were faint but distinctly present and detected by an untrained unbiased person. However, the viral loads in individual sand flies might be less than the sensitivity of the assay, and multiple infected flies could be needed for a positive result. This is a limitation of visually scored dipstick assays. The presence of homogenized sand flies in the virus positive samples did not produce false negative results.

Viral loads in wild caught *Lutzomyia* and *Phlebotomus* are poorly known; however, a study of laboratory-infected *Phlebotomus* and *Lutzomyia* indicated that the titers of SFNV and TOSV can range from 10^{3}–$10^{4.5}$ and 10^{3}–$10^{4.7}$ PFU per insect, respectively, 5–7 days postinfection (Tesh and Modi 1984). The SSFVA dipstick assay detected the target viruses (TOSV and SFNV) in a laboratory environment; however, field testing is needed to determine if this assay will be useful for threat assessments of these phlebotomine-borne viruses. The incidental ability of the SFFVA dipstick assay to detect Arumowot and the New World SF phleboviruses listed above may prove useful in detecting infected sand flies where those viruses are known to circulate. In addition, the viral antigens presented in cell culture could be different than those in infected sand flies. The SFFVA might detect more or less virus in wild infected flies. The proprietary grinding solution provided with the kit will inactivate most arboviruses, preventing them from being isolated in cell culture. However, dipstick assays are used as field screening kits for decision making, and isolation or RT-PCR might not be an option.

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REFERENCES CITED

Alkan C, Bichaud L, de Lamballerie X, Alten B, Gould EA, Charrel RN. 2013. Sandfly-borne phleboviruses of Eurasia and Africa: epidemiology, genetic diversity, geographic range, control measures. *Antiviral Res* 100:54–74.

Braito A, Corbisiero R, Corradini S, Marchi B, Sancasciani N, Fiorentini C, Ciufolini MG. 1997. Evidence of Toscana virus infections without central nervous system involvement: a serological study. *Eur J Epidemiol* 13:761–764.

Brett-Major DM, Claborn DM. 2009. Sand fly fever: what have we learned in one hundred years? *Mil Med*, 174:426–431.

Calisher CH, McLean RG, Smith GC, Szmyd DM, Muth DJ, Lazuick JS. 1977. Rio Grande—a new phlebotomus fever group virus from south Texas. *Am J Trop Med Hyg* 26:997–1002.

Charrel RN, Moureau G, Temmam S, Izri A, Marty P, Parola P, da Rosa AT, Tesh RB, de Lamballerie X. 2009. Massilia virus, a novel *Phlebovirus (Bunyaviridae)* isolated from sand flies in the Mediterranean. *Vector-Borne Zoonotic Dis* 9:519–530.

Dudley JP, Woodford, MH. 2002. Bioweapons, biodiversity, and ecocide: potential effects of biological weapons on biological diversity. *BioScience* 52: 583–592.

Endris RG, Tesh RB, Young DG. 1983. Transovarial transmission of Rio Grande virus (Bunyaviridae: *Phlebovirus*) by the sand fly, *Lutzomyia anthophora*. *Am J Trop Med Hyg* 32:862–864.

McMullan LK, Folk SM, Kelly AJ, MacNeil A, Goldsmith CS, Metcalfe MG, Batten BC, Albariño CG, Zaki SR, Rollin PE, Nicholson WL, Nichol ST. 2012. A new *Phlebovirus* associated with febrile illness in Missouri. *N Engl J Med* 367:834–841.

Peralta PH, Shelokov A, Brody JA. 1965. Chagres virus: a new human isolate from Panama. *Am J Trop Med Hyg* 14:146–151.

Ryan J, Dave K, Emmerich E, Fermamdez B, Turell M, Johnson J, Gottfried K, Burkhalter K, Kerst A, Hunt A, Wirtz R, Nasci R. 2003. Wicking assays for the rapid detection of West Nile and St. Louis encephalitis viral antigens in mosquitoes (Diptera: Culicidae). *J Med Entomol* 40:95–99.

Sabin AB. 1955. Recent advances in our knowledge of dengue and sandfly fever. *Am J Trop Med Hyg* 4:198–207.

Sather GE. 1970. Catalogue of arthropod borne viruses of the world. *Am J Trop Med Hyg* 19:1103–1104.

Savage HM, Godsey MS Jr, Lambert A, Panella NA, Burkhalter KL, Harmon JR, Lash RR, Ashley DC, Nicholson WL. 2013. First detection of Heartland virus (Bunyaviridae: *Phlebovirus*) from field collected arthropods. *Am J Trop Med Hyg* 89:445–452.

Srihongse S, Johnson CM. 1974. Human infections with Chagres virus in Panama. *Am J Trop Med Hyg* 23: 690–693.

Tesh RB. 1988. The genus *Phlebovirus* and its vectors. *Ann Rev Entomol* 33:169–181.

Tesh RB, Modi GB. 1984. Studies on the biology of phleboviruses in sand flies (Diptera: Psychodidae). I. Experimental infection of the vector. *Am J Trop Med Hyg* 33:1007–1016.

Tesh RB, Peralta PH, Shope RE, Chaniotis BN, Johnson KM. 1975. Antigenic relationships among phlebotomus fever group arboviruses and their implication for the epidemiology of sandfly fever. *Am J Trop Med Hyg* 24:135–144.

Tomori O, Fabiyi A. 1976. Antibodies against arboviruses in Sierra Leone. *Trop Geogr Med* 28: 239–243.